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14. ABSTRACT
Breast cancer development requires multiple mutations, some inherited and others acquired somatically, but the molecular causes of these mutations remain largely unknown. We hypothesize that the DNA cytosine deaminase APOBEC3B contributes directly to breast tumorigenesis by making both mutations and epigenetic alterations, that ultimately endow tumor cells with selective growth advantages. Aim 1 tests the genetic hypothesis by depleting endogenous APOBEC3B levels in representative breast cancer cell lines and, in comparison to control shRNA expressing cell lines, determining the distribution and pattern mutations by deep-sequencing and asking whether APOBEC3B levels impact the rate of therapy resistance in well-established xenograft mouse models. The first phases of these experiments have been completed and DNA samples have been prepared for deep sequencing. Aim 2 tests the epigenetic hypothesis by depleting endogenous APOBEC3B levels in representative breast cancer cell lines and by overexpressing APOBEC3B or a catalytically inactive control in normal breast epithelial cell lines and quantifying global and local changes in MeC content. These cell lines have been engineered as proposed and preliminary data analyses are underway. This work is significant because it will delineate a major source of mutations and epigenetic changes in breast cancer, which paves the way for new diagnostic/prognostic tests and methods to treat breast cancer by preventing the activity of this enzyme.

15. SUBJECT TERMS
APOBEC3B; DNA cytosine deamination; DNA methyl-cytosine deamination; epigenetics; mutation

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Introduction

The development of breast cancer, including late stage events such as metastasis and drug resistance, requires mutations. The origins of most of these mutations are unknown. We recently implicated the DNA cytosine deaminase APOBEC3B. Our Idea Award studies will test the hypothesis that APOBEC3B causes a genome wide hypermutable state and the hypothesis that APOBEC3B alters the epigenome by cytosine deamination and methyl-cytosine deamination mechanisms, respectively. Positive results will be significant because they will delineate a major source of mutations and epigenetic changes in breast cancer, and thereby pave the way for new diagnostic/prognostic tests and methods to treat breast cancer by preventing the activity of this enzyme.

Keywords

APOBEC3B; Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like-3 B; sometimes abbreviated A3B; one of 7 human A3 family members

C; Cytosine (a DNA and RNA base)

DNA; Deoxyribonucleic acid

ER; estrogen receptor (molecular target of the breast cancer therapeutic tamoxifen)

G; Guanine (a DNA and RNA base)

MeC; 5-methyl-cytosine (a common epigenetic modification in human DNA)

qPCR; Quantitative polymerase chain reaction

shRNA; short hairpin RNA (a molecular tool used to decrease gene expression)

SOW; Statement of Work

T; Thymine (a base typically found in DNA, but also the product of APOBEC3B-catalyzed MeC deamination)

U; Uracil (a base typically found in RNA but also the product of APOBEC3B-catalyzed C deamination)

Overall Project Summary

This section provides a narrative of year 1 progress. Please see **Table 1** below for an updated SOW including brief reports of the current status of each task. The original specific aims are unchanged, and studies are on-track to be completed within the proposed timeline. One minor change in experimental plan is described in Aim 1B, below, but this is expected to enhance the overall impact of the proposed studies because we will be able to determine the nature of the resistance mutations.

Aim 1 – Does A3B cause a genome-wide hypermutable state?

Aim 1 rationale: Although we have demonstrated APOBEC3B up-regulation in tumors and APOBEC3B activity in the nuclear extracts of several breast cancer cell lines[1], we still need to overcome the highest hurdle and demonstrate that APOBEC3B actually alters the genetic landscape of a breast cancer cell. This will be done by deep-sequencing to document the APOBEC3B-dependent contribution to the overall mutation distribution in cell lines and by performing a series of experiments with a well-established xenograft tumor model.

Aim 1 - Summary of Results, Progress and Accomplishments with Discussion.

Aim 1A – deep-sequencing cell lines: Deep-sequencing enables assessments of global mutation distributions and local nucleotide preferences. As proposed, we have completed the engineering of cell lines to express high and low levels of APOBEC3B, we have propagated the lines for many cell generations (to provide time for mutations to accumulate), and we have generated and prepared genomic DNA from subclones (**Appendix B, Figure 1**). These genomic DNA samples have been submitted to the University of Minnesota Genomic Center for deep-sequencing. Based on the current job queue, we expect to receive the data within 6-8 weeks and complete the data analyses within the originally proposed 18-month timeline.

Aim 1B – xenograft experiments in mice: Here, we will ask whether APOBEC3B-dependent events contribute to therapy resistance. As proposed, we have created APOBEC3B-high and isogenic APOBEC3B-depleted derivative breast cancer cell lines to ask whether APOBEC3B levels impact the rate of therapy resistance in xenograft nude mouse models. Preliminary xenograft experiments have been done to demonstrate tumor formation. We have demonstrated successful knockdown of APOBEC3B by qPCR and the xenograft experiments have been initiated by injecting nude mice as planned (**Appendix B, Figures 2 & 3**). To enhance the overall impact of this subaim, we have elected to work with the cell line MCF7L, which is an established model for ER+ breast cancer that responds to tamoxifen treatment[2]. The primary rationale for this experimental change is recent work demonstrating than many of the mutations that confer tamoxifen-resistance map to the ER gene[3-5]. As proposed originally, we will still be able to document tumor regression, quiescence, or growth-rate alterations as a function of treatment, and any subsequent tumor "escape" from the treatment (therapy resistance). However, this procedural change will allow us to identify many of the drug resistance mutations by sequencing and determine whether they occur, as expected, in

A3B preferred mutation motifs[1]. We are still on-track to complete xenograft experiments within the originally proposed 20-month timeframe.

Aim 2 – Does A3B impact genomic MeC levels?

Aim 2 rationale: The impetus for this aim stems from observations that the related DNA deaminases AID and APOBEC3A elicit MeC-to-T editing activity *in vitro*[6-8], and AID has been implicated in altering the MeC status of mouse germ and stem cells[9, 10]. Since AID is not expressed in normal breast epithelium or breast tumor cells and only A3B is up-regulated in breast tumors[1], we hypothesize that A3B alone has the capacity to remodel the breast cancer MeC landscape. This hypothesis will be tested here in experiments that are complementary to those described above.

Aim 2 - Summary of Results, Progress and Accomplishments with Discussion.

Aim 2A and 2C – A3B knockdown/overexpression and MeC quantification: A3B engineered (knockdown and over-expression) breast cancer cell lines have been generated and grown for up to 32 generations to provide sufficient time for MeC levels to change significantly (**Appendix B, Figure 1**). Genomic DNA has been harvested and the proposed analyses are underway. Initial global MeC quantifications by ELISA indicate no major differences, but this result does not exclude the possibility of significant local MeC changes. This will be assessed in Aim 2B below.

Aim 2B – Bisulfite-coupled deep-sequencing: To analyze the extent and local pattern of A3B-mediated MeC-to-T deamination, we will deep-sequence a select number of genomic DNA samples from above. Specifically, we will focus this analysis on pairs of tester and control samples that yielded positive data by ELISA, HPLC-MS/MS, and/or local bisulfite sequence analyses. The relevant genomic DNA samples have been prepared and are in the queue for bisulfite-coupled deep sequencing at the University of Minnesota Genomic Center. As above, based on the current job queue, we expect to receive the data within 6-8 weeks and complete the data analyses within the originally proposed 22-month timeline.

Table 1. Progress on original SOW with current status/progress highlighted in blue.		
Aim 1: Does APOBEC3B cause a genome-wide hypermutable state?		
Task	**Methods employed**	**Timeline and Status**
Engineering breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, and HCC1569 to knock-down endogenous A3B and generate control lines; generate multiple sub-clones for each line.	Molecular biology, cell culture, qRT-PCR	Months 1-6; completed as proposed
Preparation of genomic DNA from selected cell lines (likely HCC1569) prepared in the above tasks to express high or	General molecular biology techniques,	Months 6-18; DNA has been prepared

low levels of A3B. Delivery of DNA to sequencing facility for whole exome capture, deep sequencing, and data/sequence analysis.	data/sequence analysis, bioinformatics	and will be sent for sequencing within Month 13 (*i.e.*, on track as proposed)
Completion of IACUC forms for approval of animal experiments (80 NCr nude mice are proposed for the full xenograft experiment with numbers determined by power analysis – details can be found in the main text of the proposal). Once approved, the engineered cell lines described above (and in the narrative) will begin being xenografted into mice and therapies administered.	Cell culture, mouse model techniques	Months 1-5 for IACUC review, months 6-18 for animal procurement and xenograft experiments; IACUC approval has been received, the cell lines have been engineered, and the xenograft experiments are underway (*i.e.*, on track as proposed)
Tumor collection and analysis from xenografts.	Mouse model techniques, cancer-molecular biology techniques, qRT-PCR, sequence analysis	Months 16-20; on track as proposed.
Prepare data for publication. Publish manuscript.	Data analysis and writing	Months 20-24; on track as proposed.
Aim 2: Does APOBEC3B impact the genomic methyl-cytosine landscape?		
Task	**Methods**	**Timeframe**
Engineering of cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, and HCC1569 to knock-down endogenous A3B. Passage of lines from generations 2-32, with collection of DNA at generations 2, 4, 8, 16, and 32. Assessment of MeC levels using MeC ELISA kit.	Cell culture, molecular biology techniques, western blotting, qRT-PCR, ELISA	Months 1-6; completed as proposed.
In parallel with the task immediately above, the same DNA samples will be assessed for MeC content using HPLC-MS/MS, rather than ELISA.	Cell culture, molecular biology techniques, western blotting, qRT-PCR, HPLC-MS/MS	Months 2-7; completed as proposed.
Again, the same DNA samples as in the previous 2 tasks will be subjected to bisulfite sequencing to assess DNA	Cell culture, molecular biology	Months 3-12; completed as

methylation status in regions of the genome that are known to be effected by hypomethylation (see narrative for further details).	techniques, deep-sequencing western blotting, qRT-PCR, bisulfite sequencing	proposed with samples queued for bisulfite sequencing
We will engineer the non-tumorigenic cell lines MCF-10A (previously acquired from ATCC) and hTERT-HMEC (a gift from the lab of Dr. Vitaly Polunovsky) to over-express A3B by transfection with a linearized, tagged A3B-espression cassette followed by selection of stable clones. Control lines will be generated using the catalytically dead, tagged A3B-E255Q.	Cell culture, molecular biology techniques, western blotting, qRT-PCR	Months 3-12; completed as proposed.
Assessment of A3B over-expressing engineered cell lines' ability to alter the levels of MeC in the cell genome (determined by ELISA, HPLC-MS/MS, and bisulfite-sequencing).	Cell culture, cancer-molecular biology techniques, ELISA, HPLC-MS/MS, bisulfite sequencing	Months 8-16; in progress.
Bisulfite-coupled deep sequencing will be performed to quantify the levels of demethylation and identify any demethylation hot-spots and mutational spectra as a function of A3B expression. Samples sent for sequencing will be pairs of A3B high/A3B knock-down and A3B over-expressed/A3B-E255Q over-expressed DNA determined empirically from the previous aims to have positive results by ELISA, HPLC-MS/MS, and local bisulfite sequencing.	Bisulfite-coupled deep sequencing	Months 15-22; on-track as proposed with samples queued for bisulfite-coupled deep sequencing
Analysis and compilation of data. Assembly of manuscript.	Data analysis and writing	Months 20-24; on track as proposed.

Key Research Accomplishments

1) Cell lines have been constructed that inducibly express A3B (**Appendix B, Figure 1**).

2) Preliminary xenograft experiments have been done to demonstrate tumor formation by relevant cell lines (**Appendix B, Figures 2 & 3**).

Conclusion

Our studies are well underway and both aims are proceeding as planned. This work, we anticipate, will provide the breast cancer field with a molecular explanation for the profound genetic and epigenetic heterogeneity seen in breast cancers. This knowledge will hopefully allow scientists in the field to separate driver from passenger mutations, and focus on the altered tumor-suppressors and oncogenes that are truly important to tumorigenesis and metastasis.

Publications, Abstracts and Presentations

Publications:

Harris, R.S. (2013) Cancer mutation signatures, DNA damage mechanisms, and potential clinical implications. *Genome Medicine* 5:87 (3 pages). PMID: 24073723; PMCID: PMC3978439

Oral Presentations:

R.S. Harris; 8/13; "Evidence for APOBEC3B mutagenesis in multiple human cancers", Center for Molecular Medicine Norway, Oslo (Hans Prydz Distinguished Guest Lecture invited by Dr. H. Nilsen)

R.S. Harris; 10/13; "Integral roles for enzymatic DNA cytosine deamination in both antiviral innate immunity and cancer mutagenesis" University of Alberta, Edmonton, Canada (seminar invited by Dr. A. Mason)

R.S. Harris; 10/13; "Molecular and clinical impact of APOBEC3B mutagenesis in breast cancer", AACR special conference on Advances in Breast Cancer Research, San Diego, CA (talk invited by organizers)

R.S. Harris; 11/13; "Carcinogenesis fueled by enzyme-catalyzed DNA cytosine deamination", University of Virginia (talk invited by Drs. L. Hammarskjöld and D. Rekosh)

R.S. Harris; 11/13; "APOBEC3B mutagenesis in cancer", 29th Radiation Biology Center Symposium entitled: "Next generation radiation biology and beyond: New perspectives on DNA damage and repair", Kyoto, Japan (talk invited by organizers)

R.S. Harris; 11/13; "APOBEC3 proteins in antiviral immunity and carcinogenesis", Kyoto University, Kyoto, Japan (talk invited by Dr. A. Takori-Kondo)

R.S. Harris; 2/14; "APOBEC3B-catalyzed mutagenesis in human cancer", NCI Frederick (talk invited by Dr. S. LeGrice)

R.S. Harris; 2/14; "APOBEC3 DNA deaminases in retrovirus restriction and cancer mutagenesis", University of Wisconsin at Madison (talk invited by Dr. D. Evans)

R.S. Harris; 4/14; "Mechanism and impact of enzymatic DNA cytosine deamination in human cancers", Wake Forest School of Medicine, Department of Biochemistry (talk invited by Dr. F. Perrino)

R.S. Harris; 7/14; "Can mutagenesis be a biomarker and a therapeutic target?", Stratified Medicine Symposium, UK NHS, Guy's Hospital, London, England (talk invited by Dr. Andrew Tutt and the organizing committee)

Inventions, Patents and Licenses

Nothing to report.

Reportable Outcomes

Nothing to report.

Other Achievements

One Ph.D. student, Ms. Monica Akre, is being supported by this award. She passed her written and oral preliminary exams and is now focused on completing the proposed studies in Aim 1A.

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Appendices

Appendix A: Publication

Harris, R.S. (2013) Cancer mutation signatures, DNA damage mechanisms, and potential clinical implications. *Genome Medicine* 5:87 (3 pages). PMID: 24073723; PMCID: PMC3978439

Appendix B: Experimental Results

Figures 1 – 3 provide documentation of year 1 experimental progress.

Genome Medicine

RESEARCH HIGHLIGHT

Cancer mutation signatures, DNA damage mechanisms, and potential clinical implications

Reuben S Harris[1,2]

Abstract

Knowledge of cancer genomic DNA sequences has created unprecedented opportunities for mutation studies. Computational analyses have begun to decipher mutational signatures that identify underlying causes. A recent analysis encompassing 30 cancer types reported 20 distinct mutation signatures, resulting from ultraviolet light, deficiencies in DNA replication and repair, and unexpectedly large contributions from both spontaneous and APOBEC-catalyzed DNA cytosine deamination. Mutational signatures have the potential to become diagnostic, prognostic, and therapeutic biomarkers as well as factors in therapy development.

Germline versus somatic mutations

Every cancer is distinct. We are all conceived with near equal amounts of genetic information from each parent, and yet the resulting genetic blueprint is different for everyone (except identical twins). During development, copying and partitioning of DNA takes place during cell division such that every daughter cell receives a full genetic complement. Individuals can thus directly inherit mutations (known as germline mutations) that predispose to cancer later in life. Additionally, a variety of factors combine to diminish the fidelity of DNA copying, resulting in DNA alterations, termed somatic mutations, that distinguish a daughter cell from its sister or parent (Figure 1). Because each tumor is derived from a somatic cell, the repertoire of somatic mutations that accumulate in each tumor is distinct for each individual and reflects the underlying processes that contributed to its development.

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Driver versus passenger somatic mutations in cancer

A major rationale for sequencing large numbers of cancer genomes is to identify commonly mutated genes to inform diagnoses and treatments [1]. The mutations themselves range from simple base substitution to larger-scale aberrations such as translocations and copy number changes. The recurrent involvement of a single gene in cancers of the same type provides strong evidence for a mechanistic contribution at some stage of tumor development. Such genes are considered cancer drivers because their alteration is frequently required for tumor formation. Approximately 140 drivers have been identified and, given the massive amounts of existing data, only a few drivers probably remain uninvestigated [2].

As much as 90% to 99% of all mutations are considered passenger events. These mutations can be silent base substitutions in coding sequences but the majority occur in non-coding sequences. Such mutations are less likely to be biased by selective forces during tumor outgrowth and, therefore, can provide 'signatures' reflecting the original source of DNA damage and insights into causal mechanisms.

Global analyses of somatic mutations in cancer

Alexandrov and colleagues recently reported a comprehensive analysis of mutational signatures, examining nearly 5 million somatic mutations from over 7,000 tumors that represented 30 different cancer types [3]. This study was remarkable in three ways. First, it demonstrated the huge (1,000-fold) range in somatic mutation frequencies in human cancers. Second, computational methods enabled the deduction of over 20 distinct mutational signatures. Third, the mutation pattern of each cancer comprised at least two, and in many instances three or more, distinct mutational signatures and therefore major sources of DNA damage. Some of the DNA damage mechanisms are already established, some can be inferred based on current knowledge, and others will require more work to be fully understood.

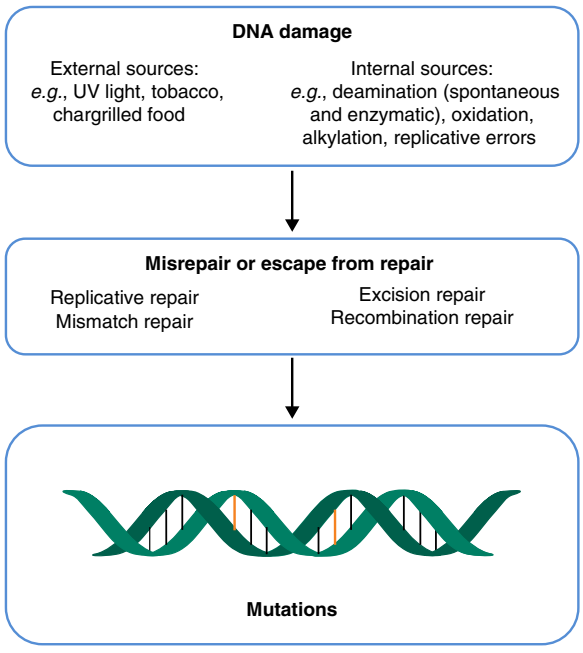

Figure 1 External and internal sources of mutation in cancer. A schematic depiction of major external and internal sources of DNA damage, a variety of DNA repair mechanisms that serve to counteract damage, and mutation as an outcome of unrepaired DNA damage.

Cancer mutation signatures from external sources of DNA damage

A major external source of DNA damage is ultraviolet (UV) light, which can crosslink adjacent pyrimidine bases (CC, CT, TC and TT) [4] (Figure 1). If such a pyrimidine dimer is not repaired and becomes a substrate for DNA replication (or local synthesis), then most DNA polymerases will follow the 'A-rule' and insert two adenines opposite the dimer. Late repair or another round of replication can then immortalize the original lesion as a C-to-T transition mutation. Thus, the mutational signature of UV light is predominantly C-to-T transitions in dipyrimidine contexts. Other features of UV-induced mutagenesis include the occurrence of adjacent mutations (mostly CC-to-TT) and a nontranscribed strand bias due to preferential repair of the transcribed DNA strand.

Tobacco smoke is another external source of DNA damage (Figure 1), but it leads to a more complex array of DNA damaging agents and lesions than UV does [5]. For instance, polycyclic aromatic hydrocarbons are converted by cellular cytochrome P450 enzymes into activated epoxides, which can then react to form alkylated guanine adducts. These lesions can erroneously base pair with adenine during DNA replication and, if unrepaired, lead to G-to-T transversions (equivalent to C-to-A on the opposing DNA strand), which comprise the most abundant class of mutations in smoking-associated cancers [3].

Many chemotherapeutics are DNA-damaging agents and, by definition, external sources of mutation. An effective chemotherapeutic should eradicate a target cancer and leave no trace for downstream analysis by sequencing. The study by Alexandrov and colleagues raises a cautionary note for treatment of glioblastomas and melanomas with the DNA methylating agent temozolomide [3]. The presence of a temozolomide-induced mutational signature in these cancers (G-to-A transition mutations at non-CpG sites) suggests not only that the intended therapy may have been ineffective but also that the drug itself may have increased the tumor mutation rate, and possibly contributed to tumor evolution, therapy resistance, and/or poor outcome. Future studies should consider mutational signatures before and after chemotherapy and strive to minimize potentially adverse outcomes.

Cancer mutation signatures from internal sources of DNA damage

Hydrolytic deamination of cytosine bases, and particularly 5-methyl-cytosine (5meC) bases in a CpG context, appears to be the most prevalent mechanism of mutagenesis [3] (Figure 1). Deamination of C-to-U or 5meC-to-T and subsequent DNA replication or misrepair results in a C-to-T transition mutation biased to CpG dinucleotide motifs. Interestingly, this is the only mechanism that correlates with age, suggesting it may be the only source of mutagenesis that accrues significantly over a lifetime [3]. Some tumors lack this signature, which suggests that these cancers might have existed for short periods and/or that they employ a mechanism of preferential repair. Other sources of chemical damage, such as oxidation, are less prevalent and may be eclipsed by more dominant mutational mechanisms.

Defects in DNA repair processes have already been linked to mutagenesis and carcinogenesis, such as in hereditary nonpolyposis colorectal cancer, which is due to inherited defects in mismatch repair [2]. Somatic inactivation and epigenetic silencing can also result in defective mismatch repair. The study by Alexandrov and colleagues confirmed the telltale signature of mismatch repair deficiency: enhanced C-to-T transitions and microsatellite instability [3]. By comparison, elevated frequencies of C-to-A transversions and C-to-T transitions occurred in a specific trinucleotide context in colorectal and uterine tumors with defects in the proofreading domain of DNA polymerase ε. In addition, an elevated frequency of insertions and deletions (without enhanced C-to-T mutagenesis) was evident in *BRCA1*- and *BRCA2*-mutant tumors, consistent with underlying defects in recombination repair.

This study also highlighted the breadth of genomic DNA deamination by members of the apolipoprotein B mRNA catalytic subunit-like (APOBEC)/activation-induced deaminase (AID) family of DNA cytosine deaminases [3]

(Figure 1). These proteins catalyze the conversion of C-to-U in single-stranded DNA, which can be converted by replication into C-to-T transition mutations or by uracil DNA glycosylase into an abasic site. This lesion can then lead to a variety of mutagenic outcomes, including C-to-T transitions, C-to-G transversions, and DNA breaks that can precipitate larger-scale aberrations. Most human cells express up to nine active DNA cytosine deaminases, with one family member (AID) functioning in antibody gene diversification and most family members protecting against virus and transposon replication [6].

Sixteen different tumor types showed evidence of an APOBEC mutational signature, characterized by both dispersed and clustered C-to-T transitions and C-to-G transversions at TC dinucleotides [3]. Mutation clusters, also called kataegis, implicated extended regions of single-stranded DNA, the preferred substrate of these enzymes. Two B cell cancers had an APOBEC signature and an additional signature consistent with AID activity [3]. Prior studies have converged upon APOBEC proteins, particularly APOBEC3B, as a major source of mutation in several types of cancer [7-9]. Because this mutational signature was similar across all sixteen tumor types, it is likely that APOBEC3B is broadly involved in cancer mutagenesis. However, additional studies are needed to assess whether one or more of the APOBEC family members may also be involved. An additional intriguing possibility, given the innate immune function of APOBEC3B and other family members, is that parasite infection may contribute to their induction and/or aberrant regulation. In terms of overall impact, APOBEC involvement in cancer mutagenesis is second only to spontaneous deamination of cytosine and 5meC [3].

Epidemiological, translational, and clinical implications

Each of the cancers studied by Alexandrov and colleagues appeared to be influenced by two or more sources of DNA damage, as deduced by their mutational signatures [3]. This knowledge has a number of important implications. First, novel signatures, such as the strong C-to-A bias in neuroblastomas and T-to-C bias in glioblastomas, will spur research to determine additional DNA damage sources. The quest to account for all mutational signatures is as much a mechanistic problem as it is epidemiological. If some of the unknown signatures are due to external sources (like UV light and tobacco carcinogens), then measures should be taken to minimize exposures.

Second, mutational signatures may act as biomarkers for the underlying mechanisms, and may become diagnostic. They will likely be even more beneficial if the mutational signatures and underlying processes correlate with clinical outcomes or specific treatments, because chemotherapeutic agents may synergize with underlying DNA damage sources (for example, PARP inhibition in *BRCA*-mutant cells [10]). Finally, it is important to emphasize that most internal sources of DNA damage are unavoidable and/or due to mistakes in DNA maintenance processes. By contrast, APOBEC/AID mutagenesis is through the aberrant action of normal enzymes, which raises the additional prospect of inhibiting these enzymes to slow down rates of tumor evolution, drug resistance, and metastasis.

Abbreviations

5meC: 5-methyl-cytosine; AID: Activation-induced deaminase; APOBEC: Apolipoprotein B mRNA catalytic subunit-like; UV: Ultraviolet.

Competing interests

RSH is a cofounder of ApoGen Biotechnologies LLC.

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Appendix B – Experimental Results

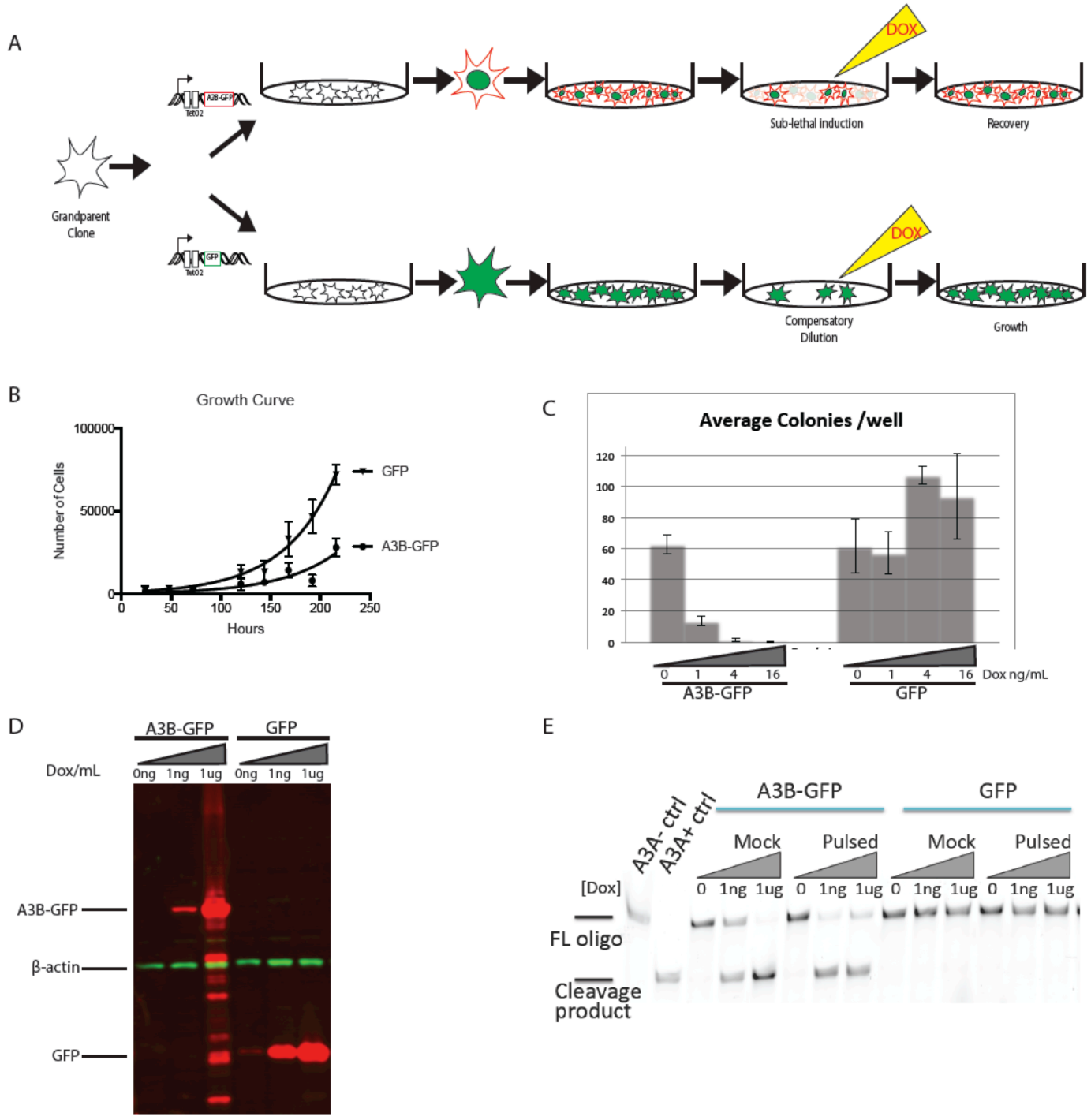

Figure 1. Inducible system for studying APOBEC3B mutagenesis. (**A**) Schematic depicting the dox-inducible 293-TRex clones engineered to express A3B-eGFP or GFP-only, and the overall workflow to study A3B mutagenesis. (**B**) Representative growth curves. (**C**) Representative titration experiment identifying 1ng/ml dox as a concentration that permits 10% survival. (**D**) Representative immunoblot showing A3B-eGFP and eGFP induction by the indicated dox concentrations (anti-β-actin serves as a loading control). (E) Representative DNA deaminase activity assay using extracts from cells treated with various concentrations of dox.

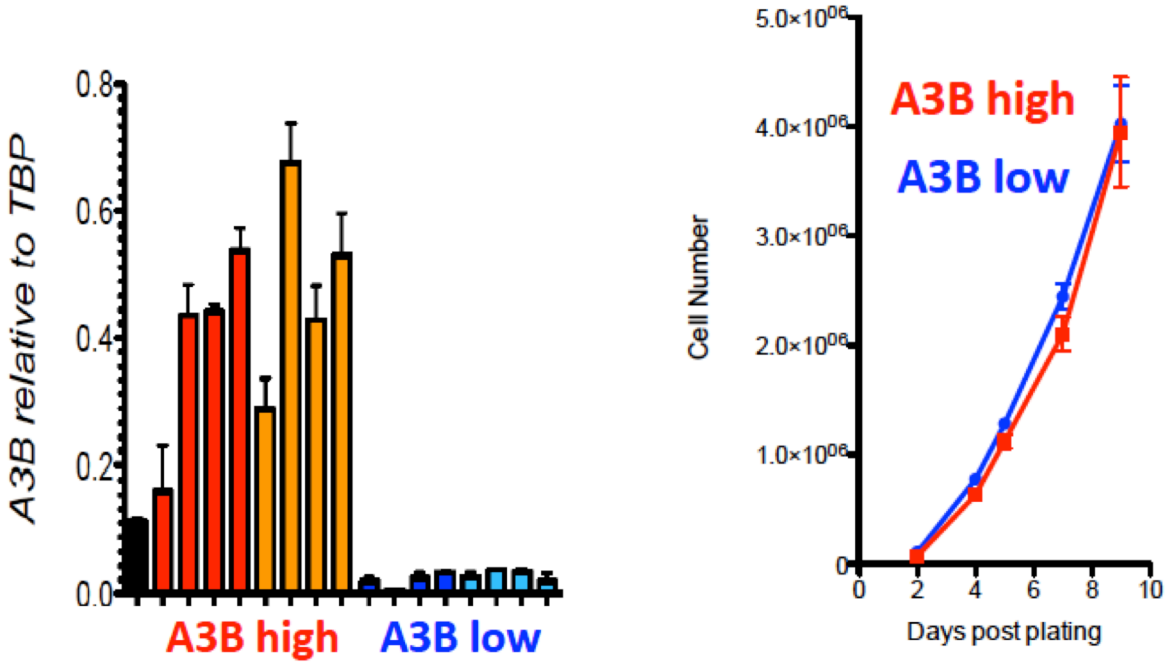

Figure 2. MCF7L cells engineered to express high and low levels of endogenous A3B. MCF7L breast cancer cells, which have naturally intermediate levels of A3B, were engineered to express a shControl construct or an shA3B construct to create derivative lines with high and low levels of endogenous A3B, respectively. The left panel shows the durability of the knockdown in cells recovered from a xenograft experiment. A similar magnitude knockdown is evident in the original pools (not shown). The right panel reprots the indistinguishable growth kinetics of MCF7L pools engineered to have high and low A3B levels. Error bars report SD of six parallel cultures for each condition.

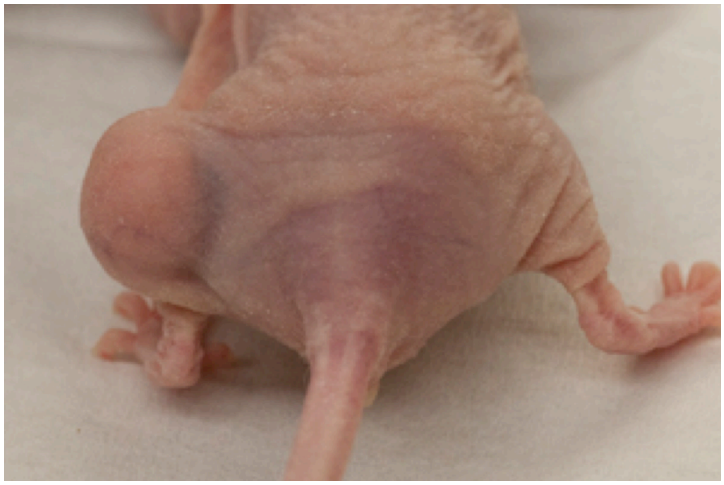

Figure 3. Tumor formation by xenografted human breast cancer cell line. Representative image of a subcutaneous tumor on the left flank of a nude and ovarectomized mouse. MCF7L breast cancer cells, which have naturally intermediate levels of A3B, were engineered to express a shControl construct or an shA3B construct to create derivative lines with high and low levels of endogenous A3B, respectively. Approximately 1 million cells were injected subcutaneously and allowed to grow *in vivo*. The animals are immune-deficient to prevent tumor rejection, and ovarectomized to force the breast cancer cells to become fully dependent on estradiol supplied in the drinking water. Tumors develop as shown within 100-150 days. Experiments are underway to evaluate the kinetics of therapy resistance using this model. Tamoxifen will be used to model estrogen receptor (ER+) tumor therapy and resistance, and estradiol-deprivation will be used to model aromatase therapy and the evolution of hormone independent growth.